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Identification of free and conjugated metabolites of mesocarb in human urine by LC-MS/MS

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Abstract The objective of the present study was to investigate mesocarb metabolism in humans. Samples obtained after administration of mesocarb to healthy volunteers were studied. The samples were extracted at alkaline pH using ethyl acetate and salting-out effect to recover metabolites excreted free and conjugated with sulfate. A complementary procedure was applied to recover conjugates with glucuronic acid or with sulfate consisting of the extraction of the urines with XAD-2 columns previously conditioned with methanol and deionized water; the columns were then washed with water and finally eluted with methanol. In both cases, the dried extracts were reconstituted and analyzed by ultra-performance liquid chromatography–tandem mass spectrometry. Chromatographic separation was carried out using a C_{18} column (100 mm×2.1 mm i.d., 1.7 µm particle size) and a mobile phase consisting of water and acetonitrile with 0.01% formic acid with gradient elution. The chromatographic system was coupled to a mass spectrometer with an electrospray ionization source working in positive mode. Metabolic experiments were performed in multiple-reaction monitoring mode by monitoring one transition for each potential mesocarb metabolite. Mesocarb and 19 metabolites were identified in human urine, including mono-, di-, and trihydroxylated metabolites excreted free as well as conjugated with sulfate or glucuronic acid. All metabolites were detected up to 48 h after administration. The structures of most metabolites were proposed based on data from reference standards available and molecular mass and product ion mass spectra of the peaks detected. The direct detection of mesocarb metabolites conjugated with sulfate and glucuronic acid without previous hydrolysis has been described for the first time. Finally, a screening method to detect the administration of mesocarb in routine antidoping control analyses was proposed and validated based on the detection of the main mesocarb metabolites in human urine (*p*-hydroxymesocarb and *p*-hydroxymesocarb sulfate). After analysis of several blank urines, the method demonstrated to be specific. Extraction recoveries of 100.3±0.8 and 105.9±10.8 (*n*=4), and limits of detection of 0.5 and 0.1 ng mL^{-1} were obtained for *p*-hydroxymesocarb sulfate and *p*-hydroxymesocarb, respectively. The intra- and inter-assay precisions were estimated at two concentration levels, 50 and 250 ng mL^{-1}, and relative standard deviations were lower than 15% in all cases (*n*=4).

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Introduction

Mesocarb, 3-(1-methyl-2-phenylethyl)-5-[[(phenylamino) carbonyl]amino]-1,2,3-oxadiazolium (Fig. 1), has a stimulant effect on the central nervous system. It is included in the list of prohibited substances of the World Anti-Doping Agency (WADA) [1] and, therefore, antidoping laboratories have to be able to detect the administration of the drug through the analysis of the parent compound or its metabolites in urine.

The first studies on mesocarb metabolism were published by Polgár et al. in rat urine [2]. Free mesocarb (1), and free and conjugated hydroxylated metabolites (p-hydroxymesocarb 2, and dihydroxymesocarb 8) were the main products described. Amphetamine was also detected as a metabolic product of mesocarb in rat urine. Early studies of our group described p-hydroxymesocarb conjugated with sulfate (compound 11 in Table 1) as the main metabolic product in human urine using liquid chromatography with diode array detection (LC-DAD) and liquid chromatography with mass spectrometry (LC-MS) techniques [3, 4]. The detection of p-hydroxymesocarb sulfate (11) in human urine was also confirmed later [5]. Appolonova et al. [6, 7] described several mono-, di-, and trihydroxylated mesocarb metabolites using electrospray LC-MS with an ion-trap system. Recently, Vahermo et al.

[8] described the synthesis and characterization of six potential hydroxylated metabolites: hydroxymesocarb (compounds 2, 3 and 4), dihydroxymesocarb (compounds 6 and 7), and trihydroxymesocarb (compound 9). However, only metabolite 2 in free and conjugated forms and metabolite 6 were detected in the samples analyzed [8]. So, the exact structures of hydroxylated metabolites described by previous authors remain unknown.

Several analytical methods have been described for the detection of mesocarb or its main metabolite, p-hydroxymesocarb conjugated with sulfate, in human urine, including LC-DAD [3, 4], particle beam LC-MS [3], thermospray LC-MS [5, 9], electrospray LC-MS [6, 7, 10–13], and LC-time-of-flight-MS [14, 15]. There are also some methodologies using gas chromatography coupled to mass spectrometry (GC-MS) [2, 16]. However, mesocarb and its hydroxylated metabolites are thermally unstable and decompose at the injection port of GC-MS [3, 6, 11, 16].

The aim of this work was to study the metabolic profile of mesocarb in human urine, including free metabolites as well as metabolites conjugated with glucuronic acid and sulfate. Regarding phase I metabolites, the main objective was to identify the hydroxylation positions of mono-, di-, and trihydroxylated metabolites described previously. The availability of reference standards of some potential

Fig. 1 Structure of mesocarb and potential phase I metabolites
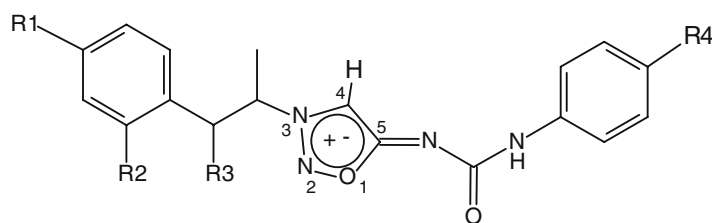
Compund code	Compound	R1	R2	R3	R4
1	Mesocarb	H	H	H	H
2	p-Hydroxymesocarb	H	H	H	OH
3	Hydroxymesocarb *(para)*	OH	H	H	H
4	Hydroxymesocarb *(ortho)*	H	OH	H	H
5	Hydroxymesocarb	H	H	OH	H
6	Dihydroxymesocarb *(para)*	OH	H	H	OH
7	Dihydroxymesocarb *(ortho)*	H	OH	H	OH
8	Dihydroxymesocarb	H	H	OH	OH
9	Trihydroxymesocarb	OH	OH	H	OH
10	Trihydroxymesocarb	OH	H	OH	OH

Table 1 UPLC-MS/MS conditions of potential mesocarb metabolites: retention time (RT), monoisotopic mass (M), precursor ion (PI), product ion (DI), cone voltage (CV), collision energy (CE) and detection in urines from excretion studies (urine)

Code	Compound	RT (min)	M	PI (*m/z*)	DI (*m/z*)	CV (V)	CE (eV)	Urine
Free metabolites								
1	Mesocarb	8.84	322.36	323	119	20	10	Yes
2	*p*-Hydroxymesocarb	7.00	338.13	339	193	20	10	Yes
3	Hydroxymesocarb (*para*)	7.15	338.13	339	135	15	10	Yes
4	Hydroxymesocarb (*ortho*)	7.90	338.13	339	135	15	10	-
5a, 5b	Hydroxymesocarb (2 isomers)	7.60/7.72	338.13	339	177	20	10	Yes
6	Dihydroxymesocarb (*para*)	5.33	354.13	355	135	20	10	Yes
7	Dihydroxymesocarb (*ortho*)	6.06	354.13	355	135	20	10	-
8a, 8b	Dihydroxymesocarb (2 isomers)	5.84/5.93	354.13	355	193	20	10	Yes
9	Trihydroxymesocarb	4.93	370.12	371	151	15	10	-
10a, 10b	Trihydroxymesocarb (2 isomers)	4.43/4.52	370.12	371	193	20	10	Yes
Sulfate conjugates								
11	*p*-Hydroxymesocarb sulfate	6.13	418.09	419	119	15	10	Yes
12	Hydroxymesocarb sulfate	6.00	418.09	419	177	20	10	Yes
13	Dihydroxymesocarb sulfate	4.94	434.08	435	135	20	10	Yes
14a, 14b	Dihydroxymesocarb sulfate (2 isomers)	5.46/5.56	434.08	435	273	15	10	Yes
Glucuronide conjugates								
15	*p*-Hydroxymesocarb glucuronide	5.88	514.16	515	369	20	10	Yes
16	Hydroxymesocarb glucuronide	5.87	514.16	515	177	20	10	Yes
17	Hydroxymesocarb glucuronide	6.09	514.16	515	177	20	10	Yes
18	Dihydroxymesocarb glucuronide	3.94	530.16	531	135	20	10	Yes
19	Trihydroxymesocarb glucuronide	4.02	546.15	547	151	20	10	Yes

metabolites makes structural elucidation possible. The final goal is to have deep knowledge on mesocarb metabolism to be able to demonstrate the illegal mesocarb administration in doping control analyses.

Experimental

Chemicals and reagents

Standards of *p*-hydroxymesocarb (**2**) and *p*-hydroxymesocarb sulfate (**11**) were supplied by United Medix laboratories Ltd. (Helsinki, Finland). Reference standards of hydroxymesocarb **3** and **4**, dihydroxymesocarb **6** and **7**, and trihydroxymesocarb **9** were supplied by University of Helsinki (Finland) [8]. 7-Propyltheophylline, synthesized at IMIM-Hospital del Mar (Barcelona, Spain), was used as internal standard.

Ethyl acetate (HPLC grade), acetonitrile and methanol (LC gradient grade), formic acid (LC/MS grade) and sodium chloride, 25% ammonia, ammonium chloride (all analytical grade) were purchased from Merck (Darmstadt, Germany). Milli Q water was obtained by a Milli Q purification system (Millipore Ibérica, Barcelona, Spain).

Sample preparation

The sample preparation for screening and confirmation purposes in doping control analysis was based on a procedure previously described [4, 13]. Briefly, to 2.5 mL aliquots of urine samples 100 ng mL^{-1} of 7-propylteophylline was added and the pH was adjusted to 9.5 with ammonium chloride solution (100 μL). Then, sodium chloride (1 g) was added to promote salting-out effect and the samples were extracted with 8 mL of ethyl acetate by shaking at 40 mpm for 20 min. After centrifugation (3,500 rpm, 5 min), the organic layers were evaporated to dryness under a nitrogen stream in a water bath at 40 °C. The extracts were reconstituted with 100 μL of a mixture of deionized water: acetonitrile (90:10, *v/v*) and aliquots of 5 μL were analyzed by UPLC-MS/MS.

For metabolic studies, in order to obtain higher extraction recoveries of metabolites conjugated with glucuronic acid, a complementary sample preparation was used [17]. To 2.5 mL of urine samples 100 ng mL^{-1} of 7-propylteophylline was added. The urine samples were applied to XAD-2 columns previously conditioned with 2 mL methanol and 2 mL water. The column was washed with 2 mL water and the analytes were eluted with 2 mL

methanol. The methanolic extract was evaporated to dryness under a nitrogen stream in a water bath at 40 °C. The dry extracts were reconstituted with 100 μL of a mixture of deionized water:acetonitrile (90:10, *v*/*v*) and aliquots of 5 μL were analyzed by UPLC-MS/MS.

Ultra-performance liquid chromatography–tandem mass spectrometry

Chromatographic separations were carried out on a Waters Acquity UPLC™ system (Waters Corporation, Milford, MA) using an Acquity BEH C_{18} column (100 mm× 2.1 mm i.d., 1.7 μm particle size). The column temperature was set to 45 °C. The mobile phase consisted of deionized water with 0.01% formic acid (solvent A) and acetonitrile with 0.01% formic acid (solvent B). For screening purposes, separation was performed at a flow rate of 0.6 mL min^{-1} with the following gradient pattern: from 0 to 0.6 min, 5% B; from 0.6 to 3.8 min, to 90% B; during 0.2 min, 90% B; from 4 to 4.1 min, to 5% B; from 4.1 to 5 min, 5% B. For metabolic studies, separation was performed at a flow rate of 0.4 mL min^{-1} and using a slow gradient pattern: from 0 to 1 min, 5% B; from 1 to 16 min, to 90% B; during 1.6 min, 90% B; from 17.6 to 18 min, to 5% B; from 18 to 20 min, 5% B. The mobile phases were filtered daily using filters of 0.22 μm. The sample volume injected was 5 μL.

The UPLC instrument was coupled to a Quattro Premier XE triple quadrupole mass spectrometer (Micromass, Waters Corporation, Milford, MA.) with an electrospray (Z-spray) ionization source working in positive ionization mode. Source conditions were fixed as follows: capillary voltage, 3 kV; lens voltage, 0.2 V; source temperature 120 °C; desolvation temperature, 450 °C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 1,200 L/h. Negative ionization mode was tested, in negative mode the conditions were the same, except the capillary voltage was set at 2.5 kV. High-purity nitrogen was used as desolvation gas, and argon was used as collision gas.

Acquisition was performed in multiple-reaction monitoring (MRM) mode. The protonated molecular ion of each compound was selected as the precursor ion. MRM conditions are described in Table 1. Electrospray ionization working parameters (ionization mode, precursor and product ions, cone voltage and collision energy) were optimized for potential metabolites available as pure standards using direct infusion of individual standard solutions of the compounds (10 μg mL^{-1}) at 10 μL min^{-1} with mobile phase (50:50, A:B) at 200 μL min^{-1}. For the rest of the metabolites detected, working parameters (cone voltage and collision energy) were optimized by analysis of extracts of urine samples obtained after administration of mesocarb. For metabolic studies, product ion-scan methods were used

to obtain product ion spectra of the precursor ion of the metabolites detected.

Method validation

The following parameters were evaluated [18]: selectivity and specificity, limit of detection (LOD), intra- and inter-assay precision at two concentration levels, ion suppression effects, and extraction recovery. Selectivity and specificity were studied by the analysis of five different blank urines obtained from different healthy volunteers. The absence of any interfering substance at the retention time of the compounds of interest was verified.

The LODs were estimated by the analysis of four replicates of blank urine samples spiked with the analytes at low concentration. The signal-to-noise ratio of these samples was evaluated, and the LOD was the extrapolated concentration with a signal-to-noise ratio of 3.

Intra-assay precision was calculated after analysis of four replicates of samples spiked at two different concentrations (50 and 250 ng mL^{-1}) on the same day. Inter-assay precision was calculated after analysis of samples spiked at these two concentration levels in four different days. Precisions were measured as the relative standard deviation of the ratios of the peak areas of the compound to the internal standard.

For evaluating ion suppression, six different blank urine samples were extracted and then spiked at 50 ng mL^{-1} with the analytes to avoid losses during the extraction procedure. The ion suppression was calculated by comparing the responses between these spiked extracts and a standard at the same concentration prepared in mobile phase. The standard deviation of the ion suppressions was calculated in order to evaluate the reproducibility between different urine matrices.

The extraction recovery was calculated by the analysis of four replicates of a blank urine spiked with the compound and four replicates of a blank sample to which the analytes were added after extraction of the blank matrix. The ratio of the peak areas between the analytes and the internal standard obtained from the extracted spiked samples were compared with ratios obtained for samples in which the analytes were added to extracted blank urines (representing 100% of extraction recovery).

Excretion study samples

Urine samples obtained in excretion studies involving the administration of mesocarb to healthy volunteers were obtained. The clinical protocol was approved by the Local Ethical committee (CEIC-IMAS, Institut Municipal d'Assistència Sanitària, Barcelona, Spain). A single dose of 10 mg of mesocarb (Sydnocarb®) was administered to two

healthy volunteers by oral route. The urine samples were collected before administration and up to 48 h after administration at the following collection periods: 0–4, 4–8, 8–12, 12–24, 24–36, and 36–48 h. Urine samples were stored at −20 °C until analysis and they were analyzed for all the metabolites. Blank urine samples collected from healthy volunteers were used in order to check the specificity.

Results and discussion

MS/MS fragmentation pattern of potential metabolites available as pure standards

Electrospray ionization working parameters were optimized for all potential metabolites available as pure standards. Positive and negative ion modes were tested. In our conditions, positive ion mode was selected because higher signal was obtained for the metabolites tested. Protonated molecular ions $[M + H]^+$ were obtained for all of the compounds, no formation of adduct ions was observed. The cone voltage was optimized to maximize the intensity of the protonated molecular ion, and the collision energy was adjusted to optimize the signal of the most abundant product ions. Product ion mass spectra of $[M + H]^+$ with proposed fragmentation patterns of the compounds tested are shown in Figs. 2 and 3 (compounds 2, 3, 4, 6, 7, 9 and 11).

Pseudomolecular ions $[M + H]^+$ of mesocarb and potential metabolites available as standards showed a characteristic MS/MS fragmentation pattern. As can be observed in Fig. 2a, for mesocarb (compound 1), the cleavage of the phenylisopropyl moiety in position 3 of the sydnone ring resulted in ion m/z 91 (tropylium ion), and ion m/z 119 is formed by cleavage of both the phenylisopropyl moiety in position 3 and the phenylcarbamoyl moiety in position 5 of the sydnone ring [2, 6, 7]. The ion at m/z 177 is formed by fragmentation of the sydnone ring [6, 7].

For p-hydroxymesocarb (2), ions m/z 91 and 119 coming from the phenylisopropyl part are present, and ions m/z 135 and 193 result from fragmentation of the phenylcarbamoyl moiety similar to the ions m/z 119 and 177 for mesocarb, with addition of 16 u corresponding to a hydroxyl group.

For p-hydroxymesocarb sulfate (11), the characteristic ions at m/z 91 and 119 coming from the phenylisopropyl part are also present. The ion at m/z 273 results from fragmentation of the sydnone ring, and the ion at m/z 193 results from fragmentation of the sydnone ring without a sulfate group.

Regarding other potential hydroxylated metabolites in the phenyl group of the phenylisopropyl moiety, two isomers were available as pure standards with hydroxyl groups in p and o positions of the phenyl group, respectively (compounds 3 and 4) [8]. The ion m/z 91 was not present in either of the compounds, however an ion at m/z 107 appeared corresponding to the tropylium ion with a hydroxyl group. The ion m/z 107 is indicative of the presence of a hydroxyl group in the phenyl ring irrespective of the position, in contrast with fragmentation proposed in previous works [7]. In addition, the fragmention of the $[M + H]^+$ ions resulted in the formation of an ion at m/z 177, coming from the phenylcarbamoyl moiety as for mesocarb, and an ion at m/z 135 coming from the phenylisopropyl moiety with an hydroxyl group.

Two potential dihydroxylated metabolites were available as pure standards [8] with the hydroxyl groups in p position of the phenylcarbamoyl moiety and p or o positions of the phenylisopropyl group, respectively (compounds 6 and 7). In both, the fragmentation of the $[M + H]^+$ ion (m/z 355) resulted in ions at m/z 107 and 135 (coming from the phenylisopropyl moiety) and at m/z 193 (coming from the phenylcarbamoyl moiety by fragmentation of the sydnone ring, as for p-hydroxymesocarb).

One potential trihydroxymetabolite was available as pure standard [8] with the hydroxyl groups in p position of the phenylcarbamoyl moiety and two hydroxyl groups in the phenylisopropyl moiety (in o and p position of the phenyl ring; compound 9). The fragmentation of the protonated molecular ion $[M + H]^+$ (m/z 371) resulted in the formation of ions at m/z 123 and m/z 151 with the same origin as ions m/z 107 and m/z 135 of hydroxymesocarb with an additional hydroxyl group.

Identification of metabolites in urines from excretion studies

A study of mesocarb metabolism with excretion study of urines obtained after administration of mesocarb to healthy volunteers was performed. As described in the experimental section, two sample preparation procedures were used to evaluate both free and conjugated fractions. The liquid–liquid extraction [4, 13] allows for a good recovery of free and sulfated metabolites. However, the recovery of glucuronide metabolites was low. For this reason, a second procedure was applied [17] to obtain good recoveries for all conjugated metabolites, sulfates and conjugates with glucuronic acid. A slow gradient elution was used to analyze the excretion study samples to obtain maximum resolution between metabolite peaks, as described in the experimental section.

Initial experiments by LC-MS/MS were performed in MRM mode by monitoring the transitions of all potential mesocarb metabolites described in the previous sections excreted in free form. In addition, transitions for metabolites conjugated with glucuronic acid or sulfate were also
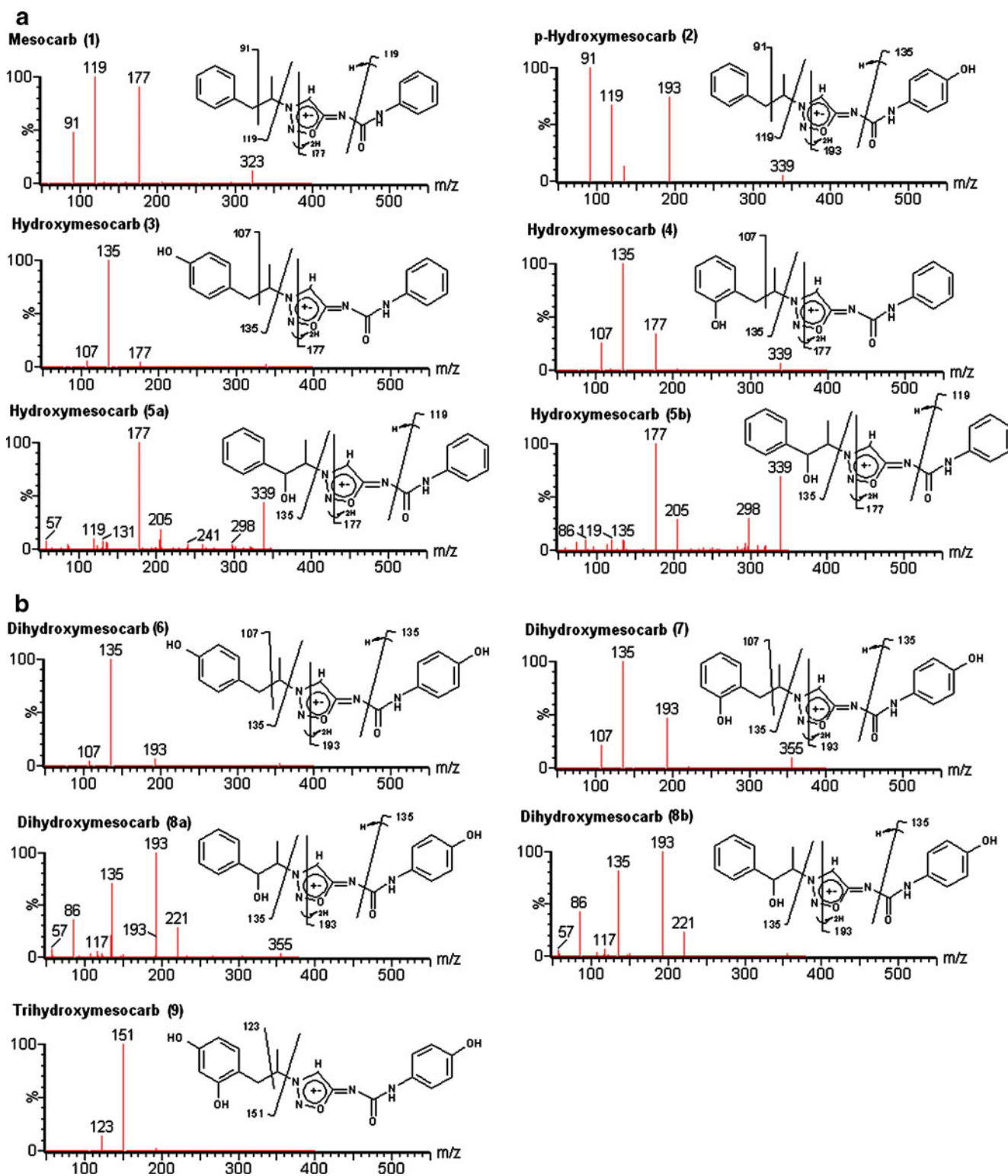
Fig. 2 Product ion mass spectra of [M + H]$^+$ and proposed fragmentation patterns of most important mesocarb metabolites excreted free in urine: **a** mesocarb and monohydroxylated metabolites; and **b** di- and trihydroxylated metabolites

tested. For conjugated metabolites, the transitions of the calculated protonated molecular ions to a characteristic product ion according to the fragmentation pattern described in previous sections were studied. Mesocarb and 19

metabolites were identified in the excretion study samples (Table 1): mono-, di-, and trihydroxylated metabolites of mesocarb were detected in free form as well as conjugated with sulfate or glucuronic acid. The optimized detection
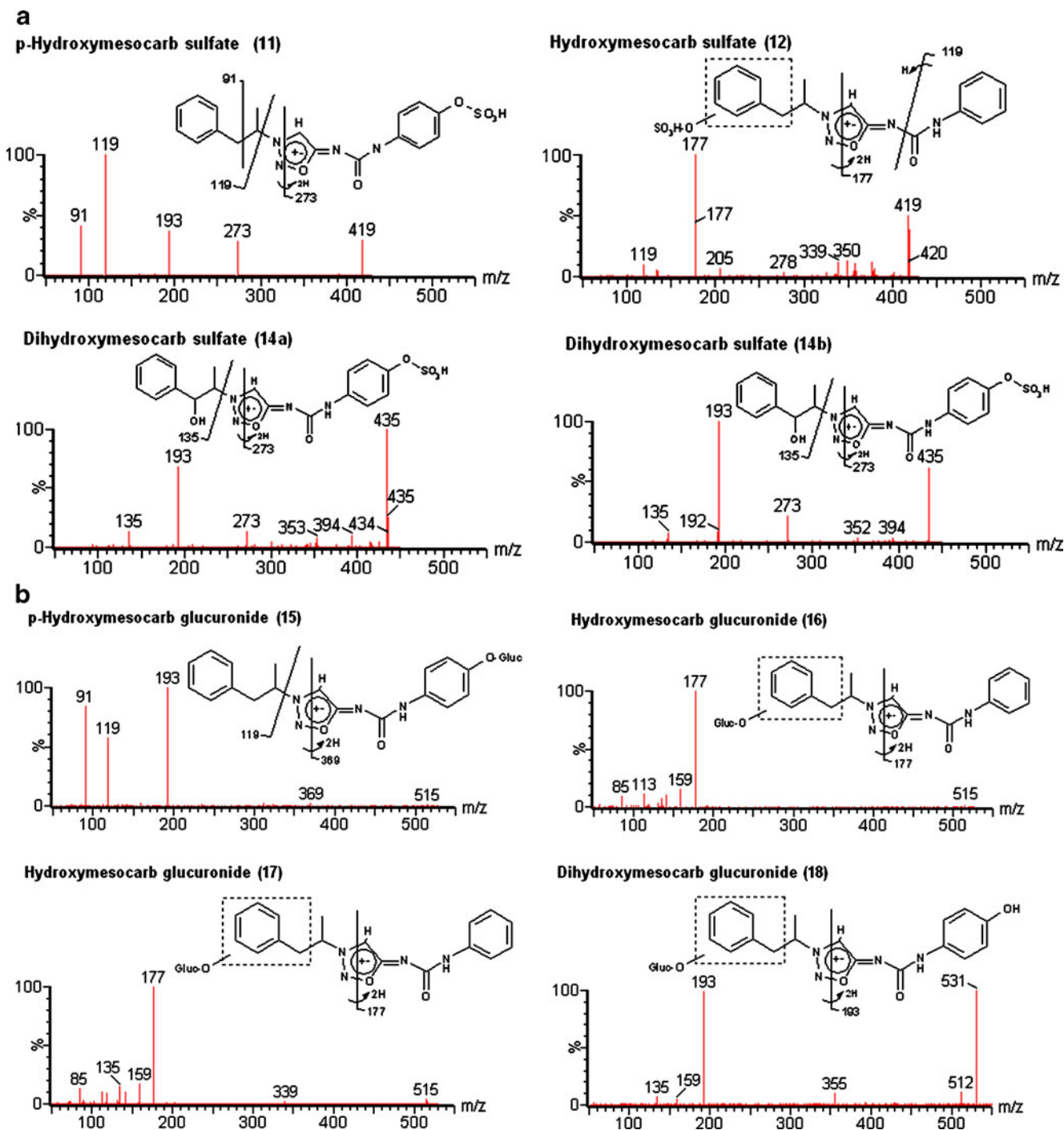
Fig. 3 Product ion mass spectra of [M + H]⁺ and proposed fragmentation patterns of the most important mesocarb metabolites excreted conjugated in urine: **a** metabolites conjugated with sulfate; and **b** metabolites conjugated with glucuronic acid. Product ion scans of metabolites 15, 16, and 17 were obtained at collision energy of 20 eV; for the rest of metabolites a collision energy of 10 eV was used as described in Table 1

conditions and retention times are listed in Table 1. Amphetamine, obtained after cleavage of the sydnone ring, identified as mesocarb metabolite in previous works [2, 6, 7] was not evaluated in our study. Chromatograms of pre- and post-administration samples are shown in Fig. 4.

After initial MRM analysis, additional analyses were performed to obtain the product ion-scan spectra of the peaks detected. Product ion-scan mass spectra were recorded when the abundance of the peak was sufficient. Product ion-scan mass spectra of [M + H]⁺ ions of

the most important metabolites are presented in Figs. 2 and 3.

Four monohydroxylated metabolites were detected in excretion study samples (**2**, **3**, **5a** and **5b**). *p*-Hydroxymesocarb was detected in the free fraction (**2**) as well as conjugated with sulfate (**11**) or glucuronic acid (**15**). Identification of metabolites **2** and **11** was performed by comparison with reference standards. *p*-Hydroxymesocarb glucuronide (**15**) was identified according to the molecular mass and the product ion mass spectra of the $[M + H]^+$ ion

of the peak (m/z 515; see Fig. 3b). Product ion mass spectra showed ions at m/z 91, 119 and 193, also present in the spectra of the metabolites **2** and **11**. An ion appeared at m/z 369, coming from the phenylcarbamoyl moiety containing the glucuronic acid group by cleavage of the sydnone ring, similar to m/z 193 and m/z 273 in the spectra of compounds **2** and **11**, respectively.

According to the MRM monitored (m/z 339 to m/z 135 or m/z 177), the hydroxyl group of the remaining three hydroxylated metabolites (**3**, **5a**, **5b**) is located in the

Fig. 4 Metabolic profile: MRM chromatograms of mesocarb metabolites: negative urine (*left*); and urine obtained 36–48 h after administration of mesocarb (*right*). **a** mesocarb and monohydroxylated metabolites excreted free in urine; **b** di- and trihydroxylated metabolites excreted free in urine; **c** metabolites conjugated with sulfate; and **d** metabolites conjugated with glucuronic acid. Chromatograms of free and sulfated metabolite were obtained after liquid–liquid extraction, and chromatograms of glucuronide metabolites were obtained after XAD-2 extraction (see "Experimental" section)
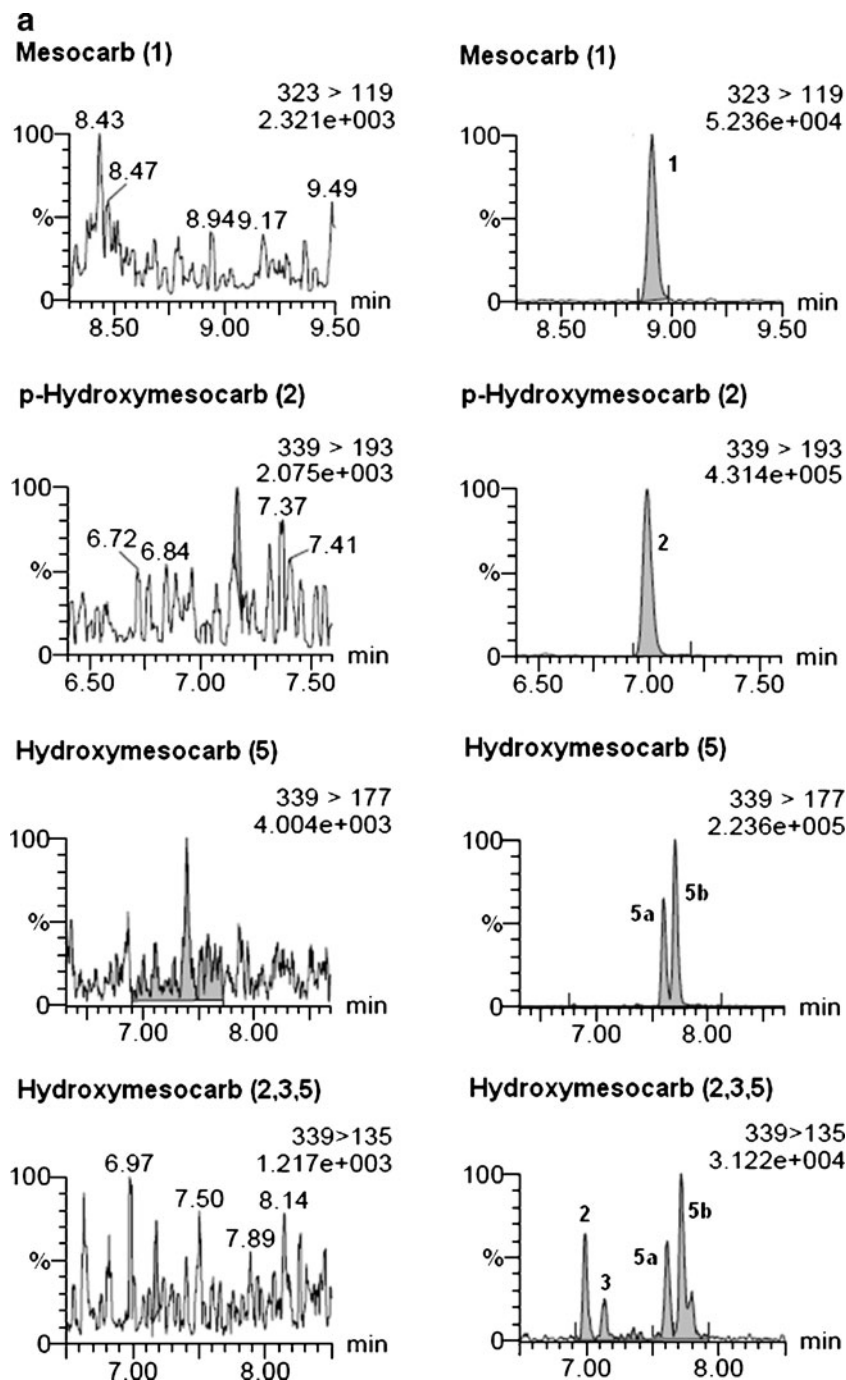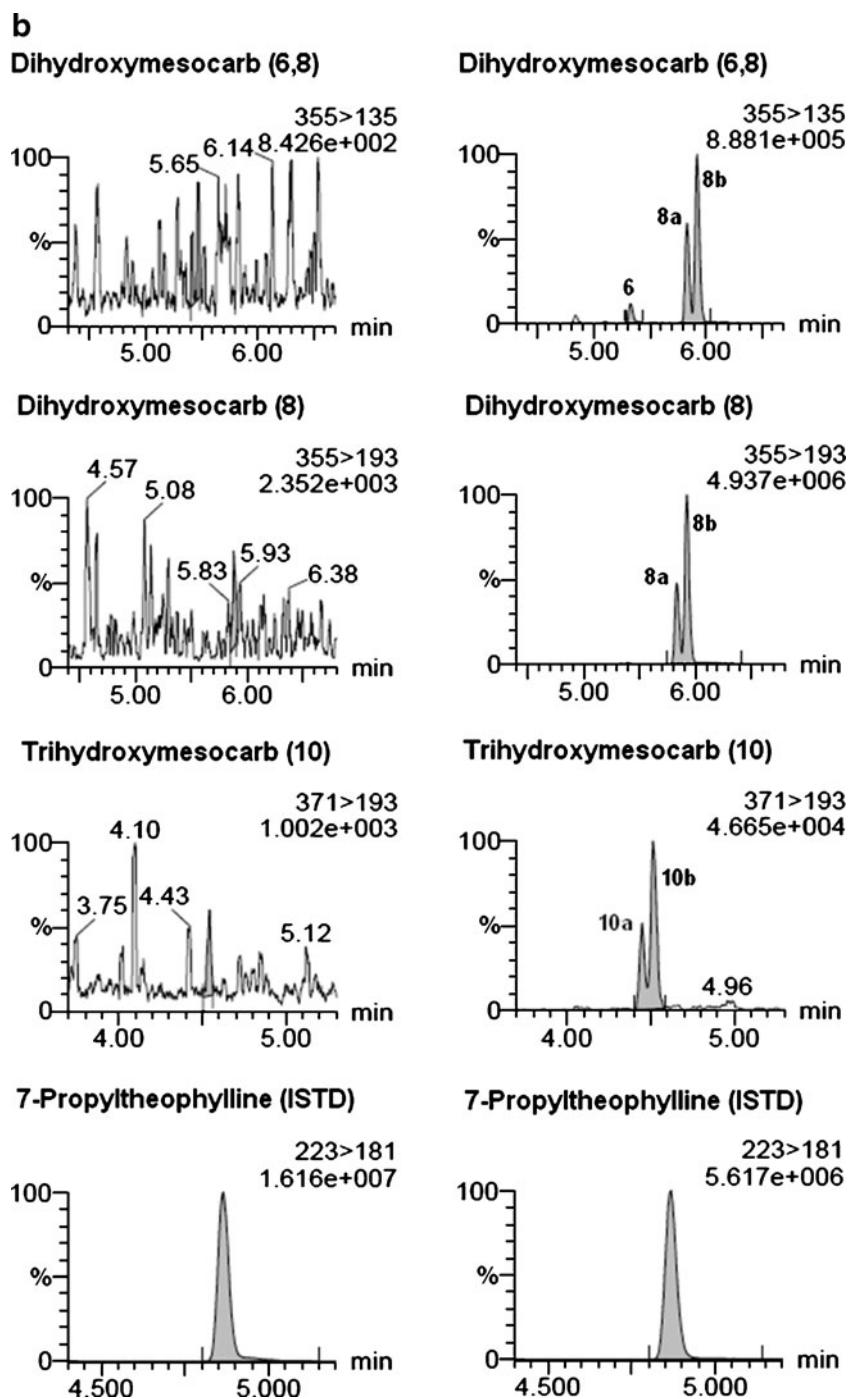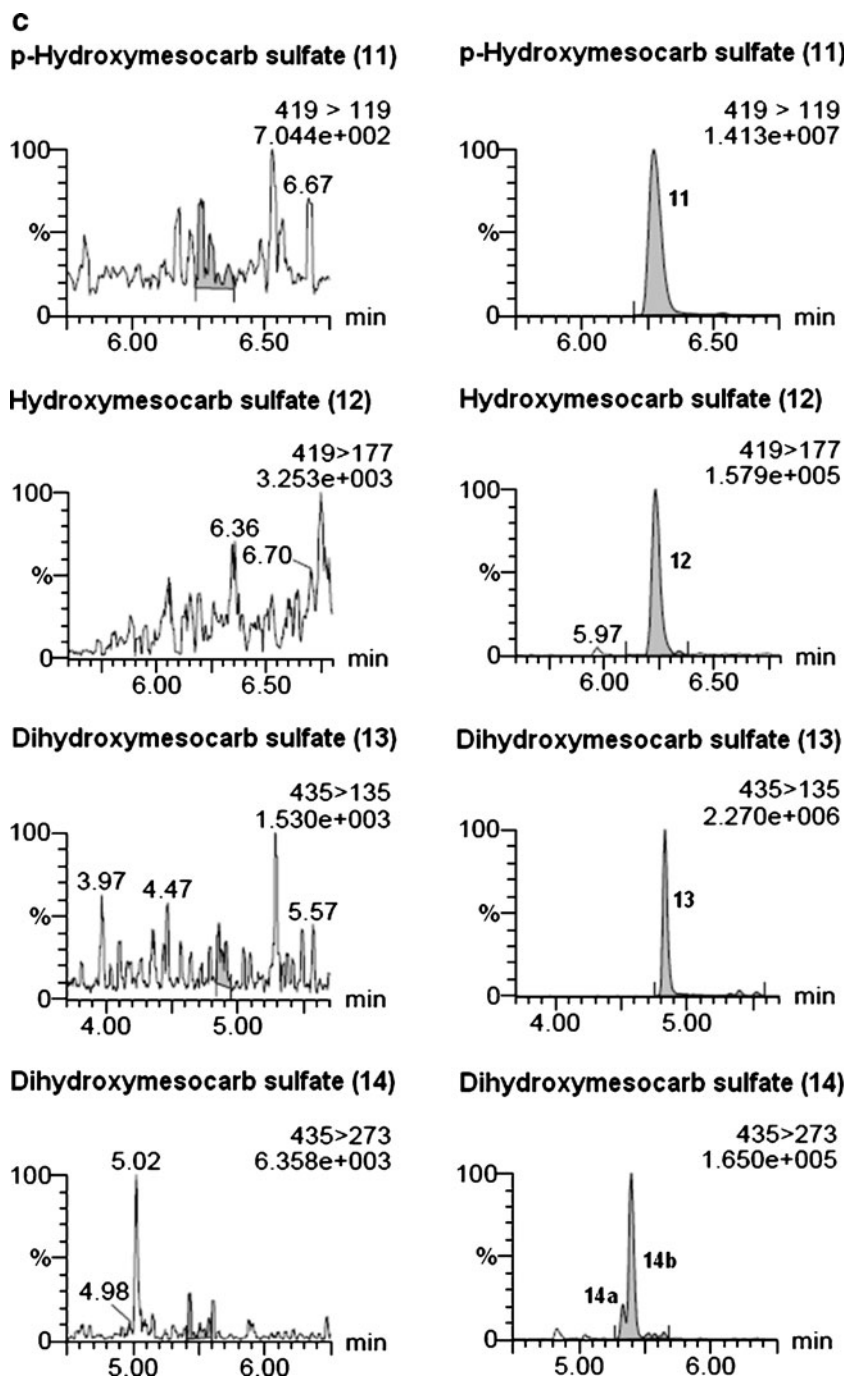
Fig. 4 (continued)

phenylisopropyl part of the mesocarb molecule. The hydroxylated metabolite in *ortho* position of the phenyl ring (compound **4**), available as pure standard, was not detected in the excretion study samples.

Identification of metabolite **3** (retention time 7.15 min) as hydroxymesocarb with hydroxyl group in *para* position of the phenyl ring was performed by comparison with the reference standard. Metabolites **5a** and **5b** showed very close retention times (7.60 and 7.72 min) and similar

product ion scan (Fig. 2a). In these product ion scans, the presence of ion at *m/z* 177 indicates that the hydroxyl group is located in the phenylisopropyl moiety; the absence of ions at *m/z* 91 is indicative of a modification in benzyl group; and the absence of ion at *m/z* 107 suggest that the hydroxyl group is not attached to the phenyl ring. According to these data, we suggest that the hydroxyl group is located at the α position of the phenylisopropyl moiety. The introduction of a hydroxyl group in position α

Fig. 4 (continued)

c

p-Hydroxymesocarb sulfate (11)

419 > 119
7.044e+002
6.67

p-Hydroxymesocarb sulfate (11)

419 > 119
1.413e+007
11

Hydroxymesocarb sulfate (12)

419>177
3.253e+003
6.36
6.70

Hydroxymesocarb sulfate (12)

419>177
1.579e+005
5.97
12

Dihydroxymesocarb sulfate (13)

435>135
1.530e+003
3.97   4.47
5.57

Dihydroxymesocarb sulfate (13)

435>135
2.270e+006
13

Dihydroxymesocarb sulfate (14)

435>273
6.358e+003
5.02
4.98

Dihydroxymesocarb sulfate (14)

435>273
1.650e+005
14b
14a

creates a second chiral center in the molecule. So, the two peaks at very close retention time may correspond to the diastereomeric metabolites.

The detection of three monohydroxylated metabolites in the phenylisopropyl part was also described in previous works, however, the hydroxylation positions were not assigned correctly [6, 7]. Based on mass spectrometric data, Appolonova et al. [6, 7] proposed that the first eluting metabolite was the α-hydroxy metabolite instead of the metabolite hydroxylated in position *para* of the phenyl ring

(compound 3). Regarding the other two metabolites, with close retention times, Appolonova et al. [6, 7] suggested hydroxylation in positions *ortho* and *para* of the phenyl ring, instead of hydroxylation in the α position (compounds **5a** and **5b** in our study). The metabolite hydroxylated in *ortho* position (compound 4) was not detected in excretion study samples. In our study, the availability of reference standards of some of the potential metabolites has allowed for the assignment of the correct structure to all these metabolites, as described in the previous paragraph.

Fig. 4 (continued)
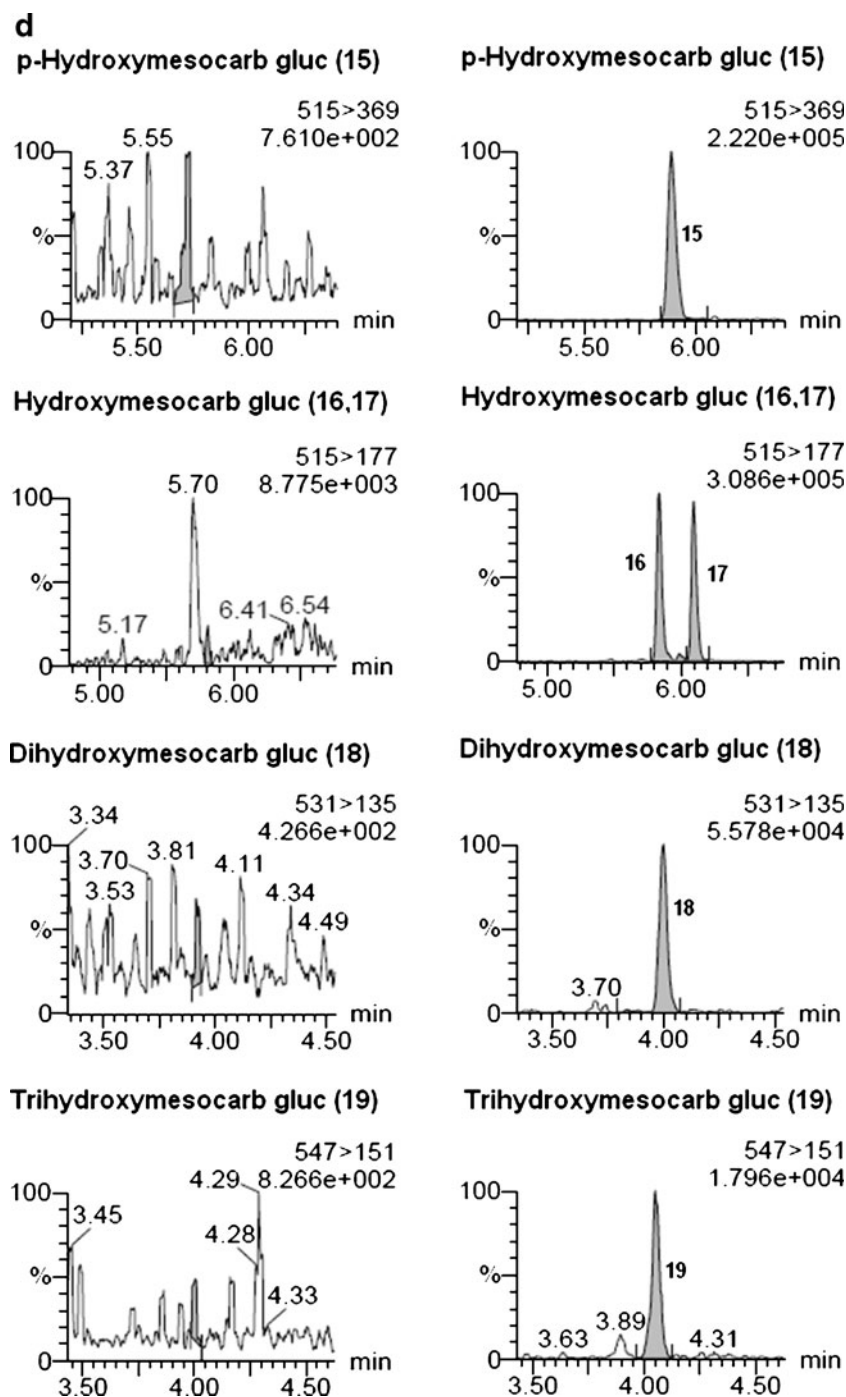
Regarding conjugates of these monohydroxylated metabolites, one peak was detected as sulfate conjugate (metabolite **12**, Fig. 3a) and two peaks were detected as conjugates with glucuronic acid (metabolites **16** and **17**, Fig. 3b). Ion at *m/z* 177, present in all product ion mass spectra of these metabolites, indicates that the phenyl-carbamoyl moiety remains unchanged as for mesocarb; thus, the conjugated hydroxyl group might be located in the phenylisopropyl part. However, these spectra do not

allow for the identification of the exact conjugated position. Because compound **4** was not detected in excretion study samples, the most feasible structure for metabolites **12**, **16**, and **17** might be the conjugation of metabolites **3**, **5a**, or **5b**.

Three dihydroxylated metabolites excreted free were identified in urines (metabolites **6**, **8a**, and **8b**). Metabolite **6** is dihydroxylated mesocarb in *para* position of both phenylisopropyl moiety and phenylcarbamoyl moiety,

available as pure standard. The second dihydroxylated metabolite available as pure standard (compound **7**) was not detected in the excretion study samples.

According to product ion mass spectra of metabolites **8a** and **8b** (Fig. 2b), one hydroxyl group is located in the phenylcarbamoyl moiety (m/z 135 and 193), and the second is located in the phenylisopropyl moiety (m/z 135). As described for monohydroxylated metabolites, we suggest that the second hydroxyl group is located in the α position of the phenylisopropyl moiety. So, a second chiral center is created and two peaks at very close retention time appear (5.84 and 5.93 min), that may correspond to the diastereomeric metabolites.

Regarding phase II metabolism, three dihydroxylated metabolites were identified as sulfate conjugates (**13**, **14a** and **14b**). Product ion mass spectra of quality could only be obtained for metabolites **14a** and **14b** (Fig. 3). Ions at m/z 193 and 273 (also appearing in metabolite **11**) indicate that these metabolites are sulfated in the hydroxyl group of the phenylcarbamoyl moiety. Additionally, the retention times of these metabolites are very close. So, we suggest that metabolites **14a** and **14b** correspond to the sulfate conjugates of metabolites **8a** and **8b**.

Two trihydroxylated metabolites were detected in the excretion study samples (**10a** and **10b**). These peaks do not correspond to compound **9**, available as standard, hydroxylated in *para* and *ortho* positions of the phenylisopropyl moiety and *para* position of the phenylcarbamoyl part. Compound **9** was not detected in excretion study samples. No product ion mass spectra of quality could be obtained for metabolites **10a** and **10b**. However, the presence of ion at m/z 193 (transition monitored 371>193) indicates that only one of the hydroxyl groups is located in the phenylcarbamoyl moiety and the other two hydroxyl groups have to be located in the phenylisopropyl part. According to the proposed structures for mono- and dihydroxymetabolites and the close retention time of the trihydroxylated metabolites **10a** and **10b** (5.46 and 5.56 min, respectively), the most feasible structure is *p*-hydroxylation in both phenyl rings and hydroxylation

in α position. The two peaks may correspond to the diastereomeric metabolites.

Trihydroxymesocarb was not detected as sulfate conjugate. However, one trihydroxylated metabolite was detected as glucuronoconjugate (metabolite **19**). It was not possible to obtain a product ion mass spectra of quality, so the conjugation position could not be proposed.

The structure of most metabolites detected has been proposed based on mass spectrometric data and on the comparison with data obtained from the reference standards available. However, the final confirmation of the structure has to be performed by comparison with reference compounds. Thus, the synthesis of those metabolites where no reference standards are available at present would be needed for the irrefutable assignment of the structures. The results of our study allow for the identification of the hydroxylation positions of mesocarb: position *para*- of both phenyl rings, and position α of the phenylisopropyl moiety. In contrast to the suggestions of previous works [6–8], hydroxylation in *ortho*- position of the phenylisopropyl part does not appear to be a metabolic pathway for mesocarb.

The direct detection of mesocarb metabolites conjugated with sulfate or glucuronic acid is described for the first time for most of the metabolites. Only *p*-hydroxymesocarb conjugated with sulfate (**11**) was directly detected in some previous works using LC-based methods [3–5]. In other previous works, selective hydrolysis of the urine samples previous to chromatographic analysis was used to identify conjugated metabolites.

Chromatograms of the urine extracts of a blank sample are compared with those obtained from 36 to 48 h after administration of mesocarb in Fig. 4. All metabolites were detected up to 48 h after administration of a single oral dose of mesocarb. The main metabolite was confirmed to be *p*-hydroxymesocarb conjugated with sulfate (**11**) during the first 48 h, in agreement with previous works [3, 6–9]. Taking into account the chromatographic signal obtained, dihydroximesocarb **8a** and **8b** excreted free and conjugated with sulfate (metabolite **13**) are then the most important

Table 2 Screening procedure: retention times (RT) and validation data of main mesocarb metabolites

Compound	RT (min)	Extraction recovery	Intra-assay precision (n=4)		Inter-assay precision (n=4)	
		n=4 (%)	ng mL^{-1}	RSD%	ng mL^{-1}	RSD%
7-Propyltheophylline (ISTD)	2.11					
p-Hydroxymesocarb (**2**)	2.54	100.3	50	14.0	50	14.0
			250	8.0	250	7.2
p-Hydroxymesocarb sulfate (**11**)	2.40	105.9	50	1.0	50	5.2
			250	10.8	250	4.0

metabolites. In urines collected after more than 48 h post-administration, a dihydroxylated metabolite excreted free was described as the major metabolite by Appolonova et al. [6, 7]. According to our results, this metabolite is dihydroxymesocarb **8a** and **8b**.

Detection of mesocarb administration in routine antidoping control

After the study of the metabolic profile, a method for screening analysis of the administration of mesocarb was proposed based on the detection of the main mesocarb metabolites, *p*-hydroxymesocarb (**2**) and *p*-hydroxymesocarb conjugated with sulfate (**11**). These compounds were included in a screening method routinely applied in our laboratory for the detection of diuretics and other acidic compounds allowing the detection of more than 30 analytes in 5 min chromatographic time [13]. This method is based on MRM detection and one transition was selected for each mesocarb metabolite (Table 1). The method was validated according to an internal protocol [18]. Retention times and results of the validation protocol are indicated in Table 2. Chromatograms of negative and positive quality control samples are presented in Fig. S1 in the Electronic Supplementary Material. The method appeared to be selective and specific after analysis of several blank urines. The absence of any interfering substance at the retention times of the compounds of interest and ISTD was verified. The limits of detection were 0.5 and 0.1 ng mL^{-1} *p*-hydroxymesocarb sulfate and *p*-hydroxymesocarb, respectively. The ion suppression evaluated using six different blank urine samples was $52.3\pm10.5\%$ and $1.1\pm14.5\%$ for *p*-hydroxymesocarb sulfate and *p*-hydroxymesocarb, respectively. Although important ion suppression effects were observed for *p*-hydroxymesocarb sulfate, the limit of detection of this compound is low enough compared to the minimum required performance limit defined by WADA for stimulants (500 ng mL^{-1}) [19] to have significant effects in routine antidoping control. Extraction recoveries near 100% were obtained for both metabolites. The intra- and inter-assay precisions, estimated at two concentration levels (50 and 250 ng mL^{-1}), were always better than 15% expressed as the relative standard deviations of the signals obtained. The confirmation analysis after a suspicious screening result can be based on the detection of the additional mesocarb metabolites (Table 1).

Conclusion

Metabolic experiments performed have been able to confirm the presence of mesocarb and 19 metabolites

excreted free or conjugated with glucuronic acid or sulfate. Structural identification of most of them has been proposed. The direct detection of mesocarb-conjugated metabolites without previous hydrolysis is described for the first time.

The screening method proposed allows determination of the most abundant metabolites of mesocarb with a simple extraction step and a chromatographic analysis of 5 min. The identification of other metabolites can be useful for confirmation purposes in doping control analyses.

The suitability of the developed methods to detect mesocarb ingestion was demonstrated by analysis of urines obtained up to 48 h after administration of a single oral dose of mesocarb to healthy volunteers.

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